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Abstract: **Monoclonal antibodies to the genus Pseudomonas, the**
labelled antibodies, compositions and kits containing them,
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Description: **Expand full description**

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+ BACKGROUND OF THE INVENTION
+ SUMMARY OF THE INVENTION
+ DETAILED DESCRIPTION

First Claim: **Show all claims**

1. A monoclonal antibody specific for an antigen or species of Pseudomonas.2q The antibody of claim 1 specific to the antigen or species of Pseudomonas cepacia.3* The antibody of claim 1 specific to the antigen or species of Pseudomonas fluorescens.

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<p>(54) Title: MONOCLONAL ANTIBODIES AND THEIR USE</p> <p>(57) Abstract</p> <p>Monoclonal antibodies to the genus <i>Pseudomonas</i>, the labelled antibodies, compositions and kits containing them, and their use in diagnosis of antigen and treatment.</p>		

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MONOCLONAL ANTIBODIES AND THEIR USE

This invention relates to monoclonal antibodies and their use.

BACKGROUND OF THE INVENTION

5 Pseudomonas is a bacterium which is widely dispersed in nature and is a common environmental contaminant in hospitals, where it infects patients with altered defence against infection. These patients include burn patients, patients with cancer or underlying metabolic diseases and
10 those who had have tubes or lines placed into various body orifices. The use of antibiotics for the treatment of other infection often leads to Pseudomonas infection. In burn and cancer centres, Pseudomonas may cause as many as 30% of all infections. The Pseudomonas species most
15 frequently associated with human disease is Pseudomonas aeruginosa which is described in Zinsser Microbiology (17th ed.) 761-5. In some hospitals, this organism causes 10 to 20% of the nosocomial infections. It has replaced Staphylococcus aureus as the major pathogen of
20 cystic fibrosis patients and is frequently isolated from individuals with neoplastic disease or severe burns. The mechanism by which Pseudomonas aeruginosa produces disease in man is not understood.

Divisions have been made among the Pseudomonas
25 species. The more commonly known Pseudomonas members include Pseudomonas cepacia, Pseudomonas fluorescens, Pseudomonas stutzeri, Pseudomonas maltophilia and Pseudomonas aeruginosa. The serotyping of Pseudomonas is not uniform among medical scientists. The system adopted
30 herein is the Liu system, as defined by P.V.Liu, which sub-divides Pseudomonas aeruginosa into 18 serotypes. Additionally, Pseudomonas is known to produce exotoxins, many of which are poorly-defined substances with toxic potential, i.e. Lps, proteases and phospholipase and
35 others being known as exotoxin A and exoenzymes. The

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extreme specificity of antigen-antibody reactions has made it possible to recognise differences between strains of such bacterial species which are indistinguishable on the basis of other phenotypic criteria.

5 Pseudomonas is known to cause gram-negative sepsis which is a bloodstream infection. It is one of the major infectious disease problems encountered in modern medical centres. While it can be transient and self-limited, severe gram-negative sepsis constitutes a medical
10 emergency.

Pseudomonas is also known to cause urinary tract infection. Gram-negative sepsis can occur during the course of a urinary tract infection, and is occasionally fatal.

15 At the present time, the test for gram-negative sepsis involves processing blood and urine cultures and other procedures on occasion. In addition to being expensive, blood culture tests are cumbersome. They require a day, and often several days, to return results.
20 They require expert laboratory skills because of the complex nature of human blood which tends to interact non-specifically with many of the test reagents.

 Presently, in urinary tract infections, a microscopic examination is made, to determine the
25 presence of micro-organisms as a preliminary screening. The microscopic examination cannot distinguish among the gram-negative bacteria. Accordingly, a second step is a urine culture to identify the organism isolated in the urine sample. A delay in diagnosis and initiation of
30 treatment can result in serious complications.

 Thus, existing methods of detection of Pseudomonas with high accuracy in urinary tract infections or gram-negative sepsis are less than satisfactory in that they consume large amounts of expensive skilled labour
35

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and laboratory time, generally taking one and often several days before returning results.

The production of monoclonal antibodies is now a well-known procedure first described by Kohler and
5 Milstein, Eur. J. Immunol. 6 (1975) 292. While the general technique of preparing hybridomas and the resultant monoclonal antibodies is understood, it has been found that preparing a specific monoclonal antibody to a specific antigen is difficult, mainly due to the
10 degree of specificity and variations required in producing a particular hybridoma.

SUMMARY OF THE INVENTION

The present invention provides novel monoclonal antibodies for use in accurately and rapidly diagnosing
15 samples for the presence of Pseudomonas antigens and/or organisms.

Briefly stated, the present invention comprises monoclonal antibodies specific for an antigen of Pseudomonas; in particular, the antigens or species of
20 Pseudomonas cepacia, the antigens or species of Pseudomonas fluorescens, the antigens or species of Pseudomonas stutzeri, the antigens or species of Pseudomonas maltophilia, the antigens or species of Pseudomonas aeruginosa 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,
25 12, 13, 14, 15, 16, 17 and 18, and the exotoxins (as described above), e.g. exotoxin A, and exoenzymes, which may be produced by Pseudomonas aeruginosa, as well as a monoclonal antibody broadly cross-reactive with an antigen for each species (or substantially all species)
30 of the genus Pseudomonas.

The invention also comprises labelled monoclonal antibodies for use in diagnosing the presence of the Pseudomonas antigens, each comprising a monoclonal antibody against one of the above-mentioned antigens to
35 Pseudomonas or to a particular species thereof and having

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linked thereto an appropriate label. The label can be, for example, a radioactive isotope, enzyme, fluorescent compound, chemiluminescent compound, bioluminescent compound, ferromagnetic atom or particle.

5 The invention further comprises the process for diagnosing the presence of Pseudomonas antigens or organisms in a specimen, comprising contacting said specimen with the labelled monoclonal antibody in an appropriate immunoassay procedure.

10 Additionally, the invention is also directed to a therapeutic composition comprising a monoclonal antibody for an antigen of Pseudomonas and a carrier or diluent, as well as kits containing at least one labelled monoclonal antibody to an antigen of a Pseudomonas.

15 DETAILED DESCRIPTION

 The monoclonal antibodies of the present invention are prepared by fusing spleen cells from a mammal which has been immunised against the particular Pseudomonas antigen, with an appropriate myeloma cell line,
20 preferably NS0 (uncloned), P3NS1-Ag4/1, or Sp2/0 Ag14. The resultant product is then cultured in a standard HAT (hypoxanthine, aminopterin and thymidine) medium. Screening tests for the specific monoclonal antibodies are employed utilising immunoassay techniques which will
25 be described below.

 The immunised spleen cells may be derived from any mammal, such as primates, humans, rodents (i.e. mice, rats and rabbits), bovines, ovines and canines, but the present invention will be described in connection with
30 mice. The mouse is first immunised by injection of the particular Pseudomonas antigen chosen, e.g. for a period of approximately eleven weeks. When the mouse shows sufficient antibody production against the antigen, as determined by conventional assay, it is given a booster
35 injection of the appropriate Pseudomonas antigen, and

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then killed so that the immunised spleen may be removed. The fusion can then be carried out utilising immunised spleen cells and an appropriate myeloma cell line.

The fused cells yielding an antibody which gives a positive response to the presence of the particular Pseudomonas antigen are removed and cloned utilising any of the standard methods. The monoclonal antibodies from the clones are then tested against standard antigens to determine their specificity for the particular Pseudomonas antigen. The monoclonal antibody selected, which is specific for the particular Pseudomonas antigen or species, is then bound to an appropriate label.

Amounts of antibody sufficient for labelling and subsequent commercial production are produced by the known techniques, such as by batch or continuous tissue culture or culture in vivo in mammals such as mice.

The monoclonal antibodies may be labelled with various labels, as exemplified above. The present invention will be described with reference to the use of an enzyme-labelled monoclonal antibody. Examples of enzymes utilised as labels are alkaline phosphatase, glucose oxidase, galactosidase, peroxidase and urease.

Such linkage with enzymes can be accomplished by any known method, such as the Staphylococcal Protein A method, the glutaraldehyde method, the benzoquinone method, or the periodate method.

Once the labelled monoclonal antibody is formed, testing is carried out employing one of a wide variety of conventional immunoassay methods. The particular method chosen will vary according to the monoclonal antibody and the label chosen. At the present time, enzyme immunoassays are preferred owing to their low cost, reagent stability, safety, sensitivity and ease of procedure. One example is the enzyme-linked immunosorbent assay (EIA). EIA is a solid-phase assay

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system which is similar in design to the radiometric assay, but which utilises an enzyme in place of a radioactive isotope as the immunoglobulin marker.

Fluorescent-immunoassay is based on the labelling of
5 antigen or antibody with fluorescent probes. A non-labelled antigen and a specific antibody are combined with identical fluorescently-labelled antigen. Both labelled and unlabelled antigen compete for antibody binding sites. The amount of labelled antigen bound to
10 the antibody is dependent upon, and therefore a measurement of, the concentration of non-labelled antigen. Examples of this particular type of fluorescent-immunoassay include heterogeneous systems such as Enzyme-Linked Fluorescent Immunoassay, or
15 homogeneous systems such as the Substrate-Labelled Fluorescent Immunoassay. The most suitable fluorescent probe, and the one most widely used, is fluorescein. While fluorescein can be subject to considerable interference from scattering, sensitivity can be
20 increased by the use of a fluorometer optimised for the probe utilised in the particular assay, and in which the effect of scattering can be minimised.

In fluorescence polarisation, a labelled sample is excited with polarised light and the degree of
25 polarisation of the emitted light is measured. As the antigen binds to the antibody, its rotation slows down and the degree of polarisation increases. Fluorescence polarisation is simple, quick and precise. However, at the present time, its sensitivity is limited to the
30 micromole per litre range and upper nanomole per litre range with respect to antigens in biological samples.

Luminescence is the emission of light by an atom or molecule as an electron is transferred to the ground state from a higher energy state. In both
35 chemiluminescent and bioluminescent reactions, the free

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energy of a chemical reaction provides the energy required to produce an intermediate reaction or product in an electronically-excited state. Subsequent decay back to the ground state is accompanied by emission of
5 light. Bioluminescence is the name given to a special form of chemiluminescence found in biological systems, in which a catalytic protein or enzyme, such as luciferase, increases the efficiency of the luminescent reaction. The best known chemiluminescent substance is luminol.

10 A further aspect of the present invention is a therapeutic composition comprising one or more of the monoclonal antibodies to the particular Pseudomonas antigen or species, as well as a pharmacologically-acceptable carrier or diluent. Such compositions can be
15 used to treat humans and/or animals afflicted with some form of Pseudomonas infection and they are used in amounts effective to cure; the amount may vary widely, depending upon the individual being treated and the severity of the infection.

20 One or more of the monoclonal antibodies can be assembled into a diagnostic kit for use in diagnosing for the presence of an antigen, antigens or species of Pseudomonas in various specimens. It is also possible to use the broadly cross-reactive monoclonal antibody which
25 can identify the genus Pseudomonas alone or as part of a kit containing antibodies that can identify other bacterial genera or species of Pseudomonas and/or other bacteria.

In the past, there have been difficulties in
30 developing rapid kits because of undesirable cross-reactions of specimens; e.g. urine with antiserum. The use of monoclonal antibodies can eliminate these problems and provide highly specific and rapid tests for diagnosis. For example, the incidence of significant
35 diarrhea and diarrheal illness is so high that estimates

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of market size for such a kit are difficult to make, but a "same day" test could be expected to be used at least as often as stool cultures. Large use of such tests in developing countries might be anticipated because of more
5 frequent and severe diarrhea, and other related illnesses.

Additionally, a kit could be used in pathology laboratories for the rapid detection of gram-negative bacteria in urine, or on an out-patient basis. Further,
10 conjugated or labelled monoclonal antibodies for antigens and/or species of Pseudomonas and other gram-negative bacteria can be utilised in a kit to identify such antigens and organisms in blood samples taken from patients for the diagnosis of possible Pseudomonas or
15 other gram-negative sepsis. The monoclonal test is an advance over existing procedures in that it is more accurate than existing tests; it gives "same day" results, provides convenience to the patient and improves therapy as a result of early, accurate diagnosis; and it
20 reduces labour costs and laboratory time required for administration of the tests.

The kit may be sold individually or included as a component in a comprehensive line of compatible immunoassay reagents sold to reference laboratories to
25 detect the species and serotypes of Pseudomonas.

One preferred embodiment of the present invention is a diagnostic kit comprising at least one labelled monoclonal antibody against a particular Pseudomonas antigen or species, as well as any appropriate stains,
30 counterstains or reagents. Further embodiments include kits containing at least one control sample of a Pseudomonas antigen and/or a cross-reactive labelled monoclonal antibody which would detect the presence of any of the given particular Pseudomonas organisms in a
35 particular sample.

Monoclonal diagnostics which detect the presence of Pseudomonas antigens can also be used in periodic testing of water sources, food supplies and food processing operations. Thus, while the present invention describes the use of the labelled monoclonal antibodies to determine the presence of a standard antigen, the invention can have many applications in diagnosing the presence of antigens by determining whether specimens, such as urine, blood, stool, water and milk, contain the particular Pseudomonas antigen. More particularly, the invention could be utilised as a public health and safety diagnostic aid, whereby specimens such as water or food could be tested for possible contamination.

The invention will be further illustrated in connection with the following Examples which are set forth for purposes of illustration only and not by way of limitation.

The monoclonal antibodies of the present invention were prepared generally according to the method of Kohler and Milstein, supra.

In the Examples:

API = Analytical Profile Index (ref. Ayerst Laboratories)

DMEM = Dulbeccos Modified Eagles Medium

FCS = Foetal Calf Serum

% T refers to vaccine concentrations measured in a 1 cm light path

PBS = Phosphate Buffered Saline

TSB = Tryptone Soya Broth

ip = intraperitoneal

iv = intravenous

im = intramuscular

CFA = Complete Freunds Adjuvant

Example 1

A. Antigen Preparation

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Pseudomonas fluorescens antigen was obtained from the National Collection of Type Cultures (NCTC accession No. 10038) and tested by standard biochemical methods of microbial identification to confirm its identity (using
5 API profiles). The Pseudomonas fluorescens was removed from the lyophile, grown on blood agar, and tested by API to confirm its identity and purity. The bacteria were transferred for growth on to TSB and harvested for use as a source of antigen. The organisms were boiled and
10 washed in formed saline by repeated centrifugation, and they were then resuspended in 1% formol saline.

B. Animal Immunisation

Balb/c mice were injected with the prepared antigen. They were given one intraperitoneal injection per week
15 for three weeks (0.05 ml 80% T vaccine), followed by 8 iv injections after intervals of 1, 4, 1, 4 x 2 and 3 weeks. The mice were bled approximately six days after the last injection and the serum tested for antibodies by assay. The conventional assay used for this serum titer testing
20 was the enzyme-linked immunosorbent assay system. When the mice showed antibody production after this regimen, generally a positive titer of at least 10,000, a mouse was selected as a fusion donor and given a booster injection (0.05 ml 80% T vaccine) intraperitoneally,
25 three days prior to splenectomy.

C. Cell Fusion

Spleen cells from the immune mice were harvested three days after boosting, by conventional techniques. First, the donor mouse selected was killed and
30 surface-sterilised by immersion in 70% ethyl alcohol. The spleen was then removed and immersed in approximately 2.5 ml DMEM to which had been added 3% FCS. The spleen was then gently homogenised in a LUX homogenising tube until all cells had been released from the membrane, and
35 the cells were washed in 5 ml 3% FCS-DMEM. The cellular

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debris was then allowed to settle and the spleen cell suspension placed in a 10 ml centrifuge tube. The debris was then rewashed in 5 ml 3% FCS-DMEM. 50 ml suspension were then made in 3% FCS-DMEM.

5 The myeloma cell line used was NS0 (uncloned), obtained from the MRC Laboratory of Molecular Biology in Cambridge, England. The myeloma cells were in the log growth phase, and rapidly dividing. Each cell line was washed using, as tissue culture medium, DMEM containing
10 3% FCS.

 The spleen cells were then spun down at the same time that a relevant volume of myeloma cells were spun down (room temperature for 7 minutes at 600 g), and each resultant pellet was then separately resuspended in 10 ml
15 3% FCS-DMEM. In order to count the myeloma cells, 0.1 ml of the suspension was diluted to 1 ml and a haemocytometer with phase microscope was used. In order to count the spleen cells, 0.1 ml of the suspension was diluted to 1 ml with Methyl Violet-citric acid solution,
20 and a haemocytometer and light microscope were used to count the stained nuclei of the cells.

10^8 spleen cells were then mixed with 6×10^7 myeloma cells, the mixture washed in serum-free DMEM high in glucose, and centrifuged, and all the liquid removed.
25 The resultant cell pellet was placed in a 37°C water-bath. 1 ml of a 50 w/v solution of polyethylene glycol 1500 (PEG) in saline Hepes, pH approximately 7.5, was added, and the mixture gently stirred for approximately 1.5 minutes. 10 ml serum-free tissue
30 culture medium DMEM were then slowly added, followed by up to 50 ml of such culture medium, centrifugation and removal of all the supernatant, and resuspension of the cell pellet in 10 ml of DMEM containing 18% by weight FCS.

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10 µl of the mixture were placed in each of 480 wells of standard multiwell tissue culture plates. Each well contains 1.0 ml of the standard HAT medium (hypoxanthine, aminopterin and thymidine) and a feeder layer of Balb/c macrophages at a concentration of 5×10^4 macrophages/well.

The wells were kept undisturbed and cultured at 37°C in 9% CO₂ air at approximately 100% humidity. The wells were analysed for growth, utilising the conventional inverted microscope procedure, after about 5 to 10 days. In those wells in which growth was present in the inhibiting HAT medium, screening tests for the specific monoclonal antibody were made utilising the conventional enzyme immunoassay screening method described below.

Somewhere around 10 days to 14 days after fusion, sufficient antibody against the Pseudomonas fluorescens antigen was developed in at least one well.

D. Cloning

From those wells which yielded antibody against the Pseudomonas fluorescens antigen, cells were removed and cloned using the standard agar method. In the agar method, a freshly-prepared stock solution of sterile 1.2% agar in double distilled water with an equal volume of double-strength DMEM and additives was maintained at 45°C. This solution (10 ml) was then aliquoted into 10 cm Petri dishes, to form a base layer. An overlay of equal volumes of agar and cells in 18% FCS-DMEM was spread evenly over the base. The cells were allowed to multiply for approximately 10 days at 37°C, 7-9% CO₂, 95% RH.

Viable separate colonies were picked off the agar surface and placed into 60 wells of a 96-well microtitre tray in 18% FCS-DMEM. After a further period of growth, the supernatants were assayed for specific antibody by the standard enzyme immunosorbent assay.

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The clones were assayed by the enzyme immunoassay method to determine antibody production.

E. Monoclonal Selection

5 The monoclonal antibodies from the clones were screened by the standard techniques for binding to Pseudomonas fluorescens NCTC 10038, prepared as in the immunisation, and for specificity in a test battery of Pseudomonas fluorescens species and related genera bearing different antigens. Specifically, a grid of
10 microtitre plates containing a representative selective of Pseudomonas organisms was prepared, boiled, and utilised as a template to define the specificity of the parent group. The EIA immunoassay noted above may be used.

15 The monoclonals had the appropriate specificity (to NCTC 10038), and were negative to other Pseudomonas, E. coli, Shigella, Salmonella, Proteus, Providencia and Serratia.

F. Antibody Production and Purification

20 Balb/c mice were primed with pristane, for at least 7 days, and injected intraperitoneally with 10^7 cells of the monoclonal antibody-producing line. The ascitic fluid was harvested when the mice were swollen with fluid but still alive. The cells were then centrifuged at 1200
25 g for approximately 10 minutes, the cells discarded, and the antibody-rich ascites collected and stored at -20 C.

Purification was accomplished using the ammonium sulphate/DEAE method.

30 In the ammonium sulphate/DEAE method, 10 ml of the ascites fluid were filtered through glass wool and centrifuged at 30,000 g for 10 minutes. The ascites was then stirred at +4°C, and an equal volume of cold, saturated ammonium sulphate added slowly. The mixture was stirred for 30 minutes after the addition was
35 complete. The precipitate was harvested by

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centrifugation at 10,000 g for 10 minutes. The precipitate was dissolved in a minimum volume of cold phosphate/EDTA buffer (20 mM sodium phosphate, 10 mM EDTA, pH 7.5, + 0.02% sodium azide). The solution was dialysed versus 2x1000 ml of the same buffer, at +4°C. The dialysed, redissolved precipitate was centrifuged at 30,000 g for 10 minutes and applied to a 10 ml column of DEAE-cellulose, previously equilibrated in phosphate/EDTA buffer. The monoclonal antibody was eluted with phosphate/EDTA buffer.

G. Enzyme-Monoclonal Linkage

The monoclonal antibody specific against Pseudomonas fluorescens antigen, prepared as above, was linked to an enzyme, viz. highly-purified alkaline phosphatase, using the one-step glutaraldehyde method. Monoclonal antibody was dialysed with alkaline phosphatase (Sigma Type VII-T) against 2 x 1000 ml of PBS pH 7.4, at +4°C. After dialysis, the volume was made up to 2.5 ml with PBS and 25 µl of a 20% solution of glutaraldehyde in PBS was added. The conjugation mixture was left at room temperature for 1.5 hours. After this time, glutaraldehyde was removed by gel filtration on a Pharmacia PD-10 (Sephadex G-25M) column, previously equilibrated in PBS. The conjugate was eluted with 3.5 ml PBS and then dialysed against 2 x 2000 ml of Tris buffer (50 mM Tris, 1 mM magnesium chloride, pH 8.0 plus 0.02% sodium azide) at +4°C. To the dialysed conjugate was added 1/10th its own volume of 10% BSA in Tris buffer. The conjugate was then sterile-filtered through a 0.22 µm membrane filter into a sterile amber vial, and stored at +4°C.

H. Monoclonal Antibody Conjugate Testing

The enzyme immunoassay method was used for testing. This method comprises coating the wells of a standard polyvinyl chloride (PVC) microtitre tray with the

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antigen, followed by addition of monoclonal antibody enzyme conjugate, and finally addition of the enzyme substrate, para-nitrophenyl phosphate.

In this case, the monoclonal antibodies were found to be specific for the antigen of Pseudomonas fluorescens. The monoclonal antibody was tested and shown to be of the Classes IgG₁.

If deemed necessary, the particular epitopic site to which the antibody attaches to the antigen can also be determined. The same enzyme immunoassay method can also be used to determine whether diagnostic specimens such as urine, blood, stool, water or milk contain the antigen. In such cases, the antibody can first be bound to the plate.

15 Examples 2 to 14

The procedure of Example 1 was followed in each of 13 cases, with differences outlined below, to prepare monoclonal antibodies and conjugates for various antigens of the genus Pseudomonas.

20 In Example 2, the antigen was Pseudomonas stutzeri, NCTC 10475; in Example 3, Pseudomonas maltophilia, NCTC 10257; in Example 4, Pseudomonas cepacia, NCTC 10743; and in Examples 5 to 13, Pseudomonas aerogenes serotypes 1 (NCTC 11440), 3 (NCTC 11442), 4 (NCTC 11443), 5 (NCTC 11444), 5d (Public Health Laboratory Service Type 5d), 6 (NCTC 11446), 8 (NCTC 11447), 9 (NCTC 11448) and 11 (PA103), respectively. Example 14 used the same antigen as Example 6 (NCTC 11442).

30 In the antigen preparation step for Example 4, blood agar was the growth medium, and a distilled water sonicate was taken. In Example 9, the growth medium was DMEM, and the organisms were boiled and washed in saline before being suspended in phenol saline. In Example 13, after growth in TSB, the supernatant was taken and the protein fraction separated.

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In the animal immunisation step for Example 2, the sequence of injections, with intervals given in numbers of weeks, was ip-1-ip-2-ip-1-iv-4-iv-1-iv-4-iv-2-iv-2-iv-8-ip. The sequence for Example 3 was ip-2-ip-2-ip-1-iv-4-iv-1-iv-2-iv-4-iv-2-iv. The sequence for Example 4 was im (in CFA)-16-iv(in formol saline). For Example 5, the sequence was ip-2-ip-2-ip-1-iv-4-iv-2-iv-2-iv-2-iv-2-iv-20-iv. For Examples 6 and 14, the sequence was ip-2-ip-2-ip-1-iv-4-iv-1-iv-2-iv-2-iv-2-iv-2-iv. For Example 7, the sequence was ip-1-ip-1-ip-1-iv-4-iv-2-iv-2-iv-2-iv-2-iv-4-iv. For Example 9, the sequence was im (in CFA)-3-ip-2-iv. The sequence for Example 10 was ip-2-ip-2-ip-1-iv-4-iv-2-iv-2-iv-20-iv. The sequence for Example 11 was ip-2-ip-2-ip-1-iv-4-iv-2-iv-2-iv-2-iv-2-iv-20-ip-1-ip. The sequence for Example 12 was ip-2-ip-2-ip-1-iv-4-iv-1-iv-2-iv-2-iv-2-iv-2-iv-7-iv-2-iv. The sequence for Example 13 was im-1-im-1-im-1-im.

In the cell fusion step, 5×10^7 myeloma cells were used in Examples 4, 5, 9, 10, 11 and 13. The number of spleen cells was 9.4×10^7 in Example 3.

In the cloning step for Examples 3, 4, 5, 6, 9, 10, 11, 13 and 14, the limiting dilution method was used instead or (in Examples 3, 6 and 14) in addition to the agar method. In limiting dilution, dilutions of cell suspensions in 18% FCS-DMEM + Balb/c mouse macrophages were made to achieve one cell/well and one-half cell/well in a 96-well microtitre plate. The plates were incubated for 7-14 days at 37 C, 97% RH, 7-9% CO₂ until semi-confluent. The supernatants were assayed for specific antibody by the standard enzyme immunoabsorbent assay.

In Examples 10, 11, 13 (in addition to the procedure given in Example 1) and 14, the antibody production step was conducted by growing cells of the monoclonal antibody-cell line in batch tissue culture. DMEM-10% FCS

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was used to support growth in mid-log phase, to 1 litre volume, and the culture was then allowed to overgrow to allow maximum antibody production. The culture was then centrifuged at 1200 g for approximately 10 minutes, the
5 cells discarded and the antibody-rich supernatant collected.

The antibody purification step for Examples 2, 3, 6, 7 and 14 involved the Protein A-Sepharose method. Ascites fluid was filtered through glass wool and centrifuged at
10 30,000 g for 10 minutes. The ascites was then diluted with twice its own volume of cold phosphate buffer (0.1M sodium phosphate, pH 8.2). The diluted ascites was applied to a 2 ml column of Protein A-Sepharose, previously equilibrated with phosphate buffer. The
15 column was washed with 40 ml of phosphate buffer. The monoclonal antibody was eluted with citrate buffer (0.1M sodium citrate, pH 3.5) into sufficient 1M TRIS buffer, pH 9.0 to raise the pH immediately to about 7.5. The eluate was dialysed in PBS, pH 7.4, at 4 C and stored at
20 -20 C.

In Examples 10 (in addition to the procedure of Example 1) and 14 (in addition to the procedures of Example 1 and the Protein A-Sepharose method given above), antibody purification was conducted by the
25 following method:

To one litre of culture supernatant was added one litre of 0.05M sodium acetate buffer, pH 4.5, and 40 ml of SP-Sephadex, previously equilibrated in 0.1M sodium acetate buffer, pH 5.0. The suspension was stirred at +4
30 C for one hour. The SP-Sephadex was allowed to settle and the supernatant decanted. The SP-Sephadex was packed in a column, washed with 60 ml of 0.1M acetate buffer, pH 5.0, and eluted with 60 ml of the same buffer plus 1M sodium chloride. The eluate was stirred at +4 C, and an
35 equal volume of saturated ammonium sulphate added slowly.

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The suspension was stirred for a further 30 minutes, and then the precipitate was harvested by centrifugation at 10,000 g for 10 minutes. The precipitate was dissolved in a minimum volume of cold phosphate/EDTA buffer (20 mM sodium phosphate, 10 mM EDTA pH 7.5 + 0.02% sodium azide). The dialysed, redissolved precipitate was centrifuged at 30,000 g for 10 minutes and applied to a 10 ml column of DEAE-cellulose, previously equilibrated in phosphate/EDTA buffer. The monoclonal antibody was eluted with phosphate/EDTA buffer.

In Example 13, antibody purification was conducted by the following method:

To one litre of culture supernatant were added 100 ml of 1.0M TRIS buffer, pH 8.2. The TRIS buffered supernatant was applied at a flow rate of 1 ml/min to a 1 ml column of Protein A-Sepharose, previously equilibrated with 0.1M TRIS buffer, pH 8.2. The column was then washed with 40 ml of 0.1M TRIS buffer. The monoclonal antibody was eluted with citrate buffer (0.1M sodium citrate, pH 3.5) into sufficient 1M TRIS buffer, pH 9.0, to raise the pH immediately to about 7.5. The eluate was dialysed in PBS, pH 7.4, at 4 C, and stored at -20 C.

The antibody conjugation step for Examples 2 and 10 was conducted using benzoquinone, as follows:

24 mg alkaline phosphatase (Sigma Type VII-T) were dialysed against 2 x 500 ml of 0.25 M sodium phosphate buffer, pH 6.0, at +4 C. 18 mg p-benzoquinone were dissolved in 0.6 ml warm AR ethanol, and added to the dialysed alkaline phosphatase. The benzoquinone/alkaline phosphatase mixture was left in the dark at room temperature for 1 hour. Unreacted benzoquinone and reaction by-products were then removed and the buffer exchanged by gel filtration on a Pharmacia PD-10 (Sephadex G-25M) column previously equilibrated in 0.15M sodium chloride. The benzoquinone-activated alkaline

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phosphatase thus produced was sufficient for six 1.5 mg antibody conjugations. Monoclonal antibody was dialysed against 2 x 500 ml of 0.15M sodium chloride at +4 C.

Dialysed antibody was added to 4 mg of

- 5 benzoquinone-activated alkaline phosphatase and immediately followed by sufficient 1M sodium bicarbonate to give a final concentration of 0.1M. The conjugation mixture was left in the dark at +4 C for 48 hours. Sufficient 1M lysine was then added to give a final
10 concentration of 0.1M. After 2 hours in the dark at room temperature, the conjugate was dialysed against 2 x 1000 ml PBS + 0.02% sodium azide at +4 C. An equal volume of glycerol was added. The conjugate was sterile-filtered through a 0.22 μ m membrane filter into a sterile amber
15 vial, and stored at +4 C.

Selection showed the appropriate specificity for each of the monoclonals of Examples 2 to 13. The monoclonals were negative to other Pseudomonas and also to E. coli (Examples 2, 6, 7 and 13), Salmonella
20 (Examples 2, 6, 7 and 13), Shigella (Examples 2, 6 and 7), Proteus (Examples 2, 3, 5, 6, 7, 10 and 12), Providencia (Examples 2, 3, 6, 7, 10 and 12), Serratia (Examples 2, 3, 7, 10, 12 and 13), Enterobacter (Example 3), Haemophilus (Example 4) and Klebsiella (Examples 6
25 and 7). The monoclonal of Example 14 was cross-reactive with Pseudomonas aeruginosa and negative to other organisms.

The Sub-classes IgG2a (Examples 2, 7, 10 and 13), IgG3 (Examples 3, 4, 6 and 11), IgM (Examples 5 and 8),
30 IgG2b (Examples 9 and 14) and IgG1 (Example 12) were found.

Example 15

The procedure of Example 1 was repeated, to give a monoclonal antibody broadly cross-reactive with each
35 species of the genus Pseudomonas.

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Tests using the present invention are superior to existing tests, based on the following advantages: (i) greater accuracy; (ii) same day results, within an hour or two; (iii) reduction in amount of skilled labour
5 required to administer laboratory procedures, resulting in reduced labour costs; (iv) reduction in laboratory time and space used in connection with tests, resulting in reduced overhead expenses; and (v) improved therapy based upon early, precise diagnosis.

10 While the invention has been described in connection with certain preferred embodiments, it is not intended to limit the scope of the invention to the particular form set forth but, on the contrary, it is intended to cover such alternatives, modifications, and equivalents as may
15 be included within the spirit and scope of the invention as defined by the appended claims.

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CLAIMS

1. A monoclonal antibody specific for an antigen or species of Pseudomonas.
2. The antibody of claim 1 specific to the antigen or species of Pseudomonas cepacia.
3. The antibody of claim 1 specific to the antigen or species of Pseudomonas fluorescens.
4. The antibody of claim 1 specific to the antigen or species of Pseudomonas stutzeri.
5. The antibody of claim 1 specific to the antigen or species of Pseudomonas maltophilia.
6. The antibody of claim 1 specific to the antigen or species of Pseudomonas aeruginosa.
7. The antibody of claim 1 specific to the antigen or species of Pseudomonas aeruginosa 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18.
8. The antibody of claim 1 specific to the exotoxins produced by Pseudomonas.
9. The antibody of claim 8 specific to the exotoxin A produced by Pseudomonas aeruginosa.
10. The antibody of claim 1 specific to an exoenzyme produced by Pseudomonas aeruginosa.
11. A monoclonal antibody broadly cross-reactive with an antigen of all species of the genus Pseudomonas.
12. A labelled monoclonal antibody consisting essentially of a monoclonal antibody of claims 1 to 11 and an appropriate label.
13. The labelled monoclonal antibody of claim 12, wherein said label is a member of the group selected from a radioactive isotope, enzyme, fluorescent compound, bioluminescent compound, chemiluminescent compound, or ferromagnetic atom, or particle.
14. The labelled monoclonal antibody of claim 13, wherein said enzyme is an enzyme capable of conjugating

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with a monoclonal antibody and of being used in an enzyme-linked immunoassay procedure.

15. The labelled monoclonal antibody of claim 14, wherein said enzyme is alkaline phosphatase, glucose
5 oxidase, galactosidase or peroxidase.

16. The labelled monoclonal antibody of claim 13, wherein said label is a fluorescent compound or probe capable of being used in an immuno-fluorescent or fluorescent immunoassay procedure, enzyme fluorescent
10 immunoassay, or fluorescence polarisation immunoassay, photon counting immunoassay, or the like procedure.

17. The labelled monoclonal antibody of claim 16, wherein said fluorescent compound or probe is fluorescein.

15 18. The labelled monoclonal antibody of claim 13, wherein said label is a chemiluminescent compound capable of being used in a luminescent or enzyme-linked luminescent immunoassay.

19. The labelled monoclonal antibody of claim 18,
20 wherein such chemiluminescent compound is luminol or a luminol derivative.

20. The labelled monoclonal antibody of claim 13, wherein said label is bioluminescent compound capable of being used in an appropriate bioluminescent immunoassay.

25 21. The labelled monoclonal antibody of claim 20, wherein such bioluminescent compound is luciferase or a luciferase derivative.

22. A process for diagnosing for the presence of an antigen of Pseudomonas in a specimen comprising
30 contacting at least a portion of said specimen with a labelled monoclonal antibody of claim 12 in an immunoassay procedure appropriate for said label.

23. The process of claim 22, wherein the appropriately labelled immunoassay procedure is selected from
35 immuno-fluorescent or fluorescent immunoassay,

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immuno-electro microscopy, radiometric assay systems, enzyme-linked immunoassays, fluorescence polarisation, photon-counting bioluminescent, or chemiluminescent immunoassay.

- 5 24. The process of claim 23, wherein said label is an enzyme capable of being used in an enzyme-linked immunoassay procedure.
25. The process of claim 24, wherein said enzyme is selected from alkaline phosphatase, glucose oxidase,
10 galactosidase or peroxidase.
26. The process of claim 23, wherein said label is a fluorescent compound or probe capable of being used in an immuno-fluorescent or fluorescent immunoassay procedure, enzyme fluorescent immunoassay, or fluorescence
15 polarisation immunoassay, or photon-counting immunoassay, or the like procedure.
27. The process of claim 26, wherein said fluorescent compound or probe is fluorescein.
28. The process of claim 23, wherein said label is a
20 chemiluminescent compound capable of being used in a luminescent or enzyme-linked luminescent immunoassay.
29. The process of claim 28, wherein said chemiluminescent compound is luminol or a luminol derivative.
- 25 30. The process of claim 23, wherein said label is a bioluminescent compound capable of being used in a bioluminescent or enzyme-linked bioluminescent immunoassay.
31. The process of claim 30, wherein said bioluminescent
30 compound is luciferase or a luciferase derivative.
32. A therapeutic composition comprising one or more of the labelled monoclonal antibodies in claims 1 to 11 and a pharmaceutically acceptable carrier or diluent.

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33. A therapeutic composition comprising one or more of the labelled monoclonal antibodies in claim 12 and a pharmaceutically acceptable carrier or diluent.
34. A method for treating Pseudomonas infections
- 5 comprising administering an effective amount of a monoclonal antibody of claims 1 to 11.
35. A kit for diagnosing for the presence of an antigen or species of Pseudomonas in a diagnostic specimen comprising at least one monoclonal antibody of claims 1
- 10 to 11.
36. The kit of claim 35, wherein said at least one antibody is labelled.
37. The kit of claim 36, wherein said at least one antibody is labelled with a fluorescent compound.
- 15 38. The kit as in claim 36, wherein said at least one monoclonal antibody is labelled with an enzyme.
39. The kit as in claim 36, wherein said at least one monoclonal antibody is labelled with a member of the group consisting of a radioactive isotope,
- 20 chemiluminescent compound, bioluminescent compound, ferromagnetic atom, or particle.
40. The kit of claims 36, 37, 38 and 39 additionally containing at least one known Pseudomonas antigen as a control.
- 25 41. The kit of claim 36, 37, 38, 39 and 40 containing each known antigen of Pseudomonas cepacia, Pseudomonas fluorescens, Pseudomonas stutzeri, Pseudomonas maltophilia and Pseudomonas aeruginosa 1 to 18 (inclusive).
- 30 42. The kit of claims 36, 37, 38, 39 and 40 containing the antigens of Pseudomonas cepacia.
43. The kit of claims 36, 37, 38, 39 and 40 containing the antigens of Pseudomonas fluorescens.
44. The kit of claims 36, 37, 38, 39 and 40 containing
- 35 the antigens of Pseudomonas stutzeri.

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45. The kit of claims 36, 37, 38, 39 and 40 containing the antigens of Pseudomonas maltophilia.
46. The kit of claims 36, 37, 38, 39 and 40 containing the antigens of Pseudomonas aeruginosa.
- 5 47. The kit of claims 36, 37, 38, 39 and 40 containing the antigens of Pseudomonas aeruginosa 1 to 18 (inclusive).
48. The kit of claims 36, 37, 38, 39 and 40 containing the exotoxins of Pseudomonas.
- 10 49. The kit of claim 48, wherein the exotoxin is exotoxin A produced from Pseudomonas aeruginosa.
50. The kit of claim 48, wherein the exotoxin is an exoenzyme produced from Pseudomonas aeruginosa.
- 15 51. A kit for diagnosing for the presence of an antigen or species of Pseudomonas in a diagnostic specimen, comprising at least one monoclonal antibody of any of claims 1 to 11, and a control.
52. The kit of claim 51, wherein said at least one antigen is labelled and said control is at least one
- 20 known antigen of Pseudomonas.
53. A kit for diagnosing for the presence of a gram-negative bacterial infection comprising at least one monoclonal antibody of any of claims 1 to 11.
54. The kit of claim 53, wherein said at least one
- 25 monoclonal antibody is labelled.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 85/00409

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁴ According to International Patent Classification (IPC) or to both National Classification and IPC IPC: ⁴ C 07 K 15/00; C 12 P 21/00; A 61 K 39/40; A 61 K 43/00; G 01 N 33/569; G 01 N 33/577 //(C 12 P 21/00, C 12 R 1:91)																				
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; border-bottom: 1px solid black;">Classification System</th> <th style="width: 70%; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;"> ⁴ IPC <div style="border-left: 1px solid black; border-right: 1px solid black; padding: 0 10px; margin-top: 5px;"> G 01 N C 12 P A 01 K </div> </td> <td></td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	⁴ IPC <div style="border-left: 1px solid black; border-right: 1px solid black; padding: 0 10px; margin-top: 5px;"> G 01 N C 12 P A 01 K </div>															
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ⁹</th> <th style="width: 60%; border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">P, X</td> <td style="vertical-align: top; padding: 5px;"> Chemical Abstracts, volume 103, no. 9, 2 September 1985, Columbus, Ohio, (US) R.E.W. Hancock et al.: "Immunotherapeutic potential of monoclonal antibodies against Pseudomonas aeruginosa protein F", see page 493, column 1, abstract no. 69460n & Eur. J. Clin. Microbiol., 1985, 224-227 (Eng) </td> <td style="vertical-align: top; padding: 5px; text-align: center;"> 1, 6, 11, 32, 34 </td> </tr> <tr> <td colspan="3" style="text-align: center; padding: 5px;">--</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">P, X</td> <td style="vertical-align: top; padding: 5px;"> Chemical Abstracts, volume 102, no. 19, 13 May 1985, Columbus, Ohio, (US) R.T. Irvin et al.: "Immunochemical examination of the Pseudomonas aeruginosa glycocalyx: a monoclonal antibody which recognizes L- guluronic acid residues of alginic acid", see page 473, column 2, abstract no. 165021v & Can. J. Microbiol., 1985, 268-275 (Eng) </td> <td style="vertical-align: top; padding: 5px; text-align: center;"> 1, 6, 12, 13, 14, 16, 22-24, 26 </td> </tr> <tr> <td colspan="3" style="text-align: center; padding: 5px;">--</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">P, X</td> <td style="vertical-align: top; padding: 5px;"> Chemical Abstracts, volume 102, no. 13, 1 April 1985, Columbus, Ohio, (US) K. Murakami et al.: "Monoclonal antibodies against species-specific </td> <td></td> </tr> </table> <div style="font-size: small; margin-top: 10px;"> <p>⁹ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	P, X	Chemical Abstracts, volume 103, no. 9, 2 September 1985, Columbus, Ohio, (US) R.E.W. Hancock et al.: "Immunotherapeutic potential of monoclonal antibodies against Pseudomonas aeruginosa protein F", see page 493, column 1, abstract no. 69460n & Eur. J. Clin. Microbiol., 1985, 224-227 (Eng)	1, 6, 11, 32, 34	--			P, X	Chemical Abstracts, volume 102, no. 19, 13 May 1985, Columbus, Ohio, (US) R.T. Irvin et al.: "Immunochemical examination of the Pseudomonas aeruginosa glycocalyx: a monoclonal antibody which recognizes L- guluronic acid residues of alginic acid", see page 473, column 2, abstract no. 165021v & Can. J. Microbiol., 1985, 268-275 (Eng)	1, 6, 12, 13, 14, 16, 22-24, 26	--			P, X	Chemical Abstracts, volume 102, no. 13, 1 April 1985, Columbus, Ohio, (US) K. Murakami et al.: "Monoclonal antibodies against species-specific	
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IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search 19th December 1985 </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report 05 FEB. 1986 </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="border-bottom: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: right;"> G.L.M. Klavdenberg </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 19th December 1985	Date of Mailing of this International Search Report 05 FEB. 1986	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer <div style="text-align: right;"> G.L.M. Klavdenberg </div>														
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	cephalosporinase of Pseudomonas aeruginosa", see page 517, column 1, abstract no. 111126q, & Eur.J.Biochem. 1985, 693-697 (Eng)	1,6,10,22
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X	Chemical Abstracts, volume 101, no. 25, 17 December 1984, Columbus, Ohio (US) S. Sawada et al.: "Protection against infection with Pseudomonas aeruginosa by passive transfer of monoclonal antibodies to lipopolysaccharides and outer membrane proteins", see page 588, column 1, abstract no. 228262b	1,6,7,11,32,34
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X	Chemical Abstracts, volume 100, no. 25, 18 June 1984, Columbus, Ohio (US) D.R. Galloway et al.: "Production and characterization of monoclonal antibodies to exotoxin A from Pseudomonas aeruginosa", see page 422, column 1, abstract no. 207654v & Infect.Immun. 1984, 262-267 (Eng)	1,6,8,9,13,14,22,23,24,32,34
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X	Chemical Abstracts, volume 100, no. 5, 30 January 1984, Columbus, Ohio (US) L.M. Mutharia et al.: "Surface localization of Pseudomonas aeruginosa outer membrane porin protein F by using monoclonal antibodies", see page 333, column 2, abstract no. 33069r & Infect.Immun., 1983, 1027-1033 (Eng)	1,6,22,23,26
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A	EP, A, 0101039 (MEIJI SEIKA KABUSHIKI KAISHA) 22 February 1984, see the whole document	1,6,7,22,32,34
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X	EP, A, 0077734 (MOLECULAR GENETICS INC.) 27 April 1983, see page 20, line 30 - page 22, line 15; claims 1-3,9,12	1,6,32,34
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A	WO, A, 83/01739 (BRIGHAM AND WOMENS HOSPITAL) 26 May 1983, see pages 3,4,14-19	13,14,16,18,22-24,26,28,30,32,34,36-39,53,54
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A	EP, A, 0105714 (SERONO DIAGNOSTICS LTD.) 18 April 1984	
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A, 0111762 (UNILEVER PLC) 27 June 1984 -----	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 85/00409 (SA 10645)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 20/01/86

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0101039	22/02/84	JP-A- 59029622	16/02/84
EP-A- 0077734	27/04/83	AU-A- 8945482	28/04/83
		JP-A- 58099423	13/06/83
		US-A- 4443549	17/04/84
		CA-A- 1187822	28/05/85
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		EP-A- 0093775	16/11/83
EP-A- 0105714	18/04/84	AU-A- 1964883	05/04/84
EP-A- 0111762	27/06/84	EP-A- 0042755	30/12/81
		WO-A- 8200058	07/01/82
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		EP-A- 0146654	03/07/85

For more details about this annex :
see Official Journal of the European Patent Office, No. 12/82